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TECHNICAL NOTE

Free cysteine is increased in plasma from hemodialysis patients

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Free cysteine is increased in plasma from hemodialysis patients.

Background. Although only the total thiol concentration, which includes bound and free forms, has been determined in most previous clinical studies, the free form may be a better predictor of cardiovascular risk.

Methods. We measured the apparent concentration of free homocysteine (Hcy) and cysteine (Cys) in filtered and acid-soluble fractions of plasma in healthy control subjects and in patients with chronic renal failure just before and after a hemodialysis session.

Results. In control, filtered Hcy and acid-soluble Hcy were similar, while filtered Cys was much smaller than in acid-soluble Cys. In prehemodialysis samples, filtered Cys was more than 60 times as abundant (259.2 ± 26.2 μmol/L) as in control samples (4.1 ± 0.7 μmol/L). Free-to-total ratios for filtered Cys were 1.6 ± 0.3% in controls, but 40.9 ± 2.7% in prehemodialysis patients.

Conclusions. The filtered fraction of thiols can be used to estimate solute transport across the dialysis membrane. In addition, the possible involvement of cysteine in the pathogenesis of atherosclerosis in hemodialysis patients should be reexamined.

The thiols homocysteine (Hcy) and cysteine (Cys) recently have received much attention, especially concerning hyperhomocysteinemia or hypercysteinemia as an independent risk factor for early-onset cardiovascular disease (CVD) in chronic renal failure patients as well as the general population [1–4]. This interest has spurred development of sensitive and specific assays using a derivatization agent [5]. While only total thiol concentrations, which include both protein-bound and free forms, have been determined in most previous studies, the free form may be a better predictive index for cardiovascular events.

Cytotoxicity from oxidative stress induced by Hcy or Cys could depend on the redox status of plasma thiol. The reduced form of thiols is likely to play the more important role in oxidative insults, damaging cells by producing hydroxyl radicals in the course of their auto-oxidation. However, since reduced aminothiols usually are unstable, results of assays are often incorrect [6]. Specifically, these reduced forms oxidize very rapidly in human plasma [7]. Measurement of free as opposed to protein-bound forms of thiols may be a good surrogate for the reduced form, as concentration changes for the reduced form should parallel the changes for the free form.

As for possible mechanisms of vascular pathogenesis apart from oxidative stress, adverse intimal extracellular matrix (ECM) remodeling and also disruption of some intracellular processes may follow cellular uptake of unbound forms of aminothiols [8], whether reduced or in the disulfide form. We, therefore, focused on the free forms of aminothiols.

Previous investigations attempted to determine the free forms of thiols in acid-precipitated samples (acid-soluble fraction) [9, 10]. In estimating renal handling of thiols or their removal by dialysis, acid precipitation is not a suitable method since acidity may affect both thiol binding and disulfide bonds. Acid precipitation can liberate bound thiols and other substances from proteins, particularly albumin. In the present study, we analyzed free thiols in a fraction of plasma passing through a filter used as a molecular sieve and compared free thiols in the filtered sample to free thiols in the acid-soluble fraction.

METHODS

Study patients

Twenty patients undergoing maintenance hemodialysis (10 men, 10 women; age, 46.0 ± 3.0 years) were recruited for the present study. Hemodialysis was performed for 4 hours three times a week using polysulfone (BS series; Toray, Tokyo, Japan) or cellulose triacetate membranes (FB series; Nipro, Osaka, Japan). Blood samples were collected into sterile tubes by venipuncture. In patients undergoing hemodialysis, blood was sampled before and after hemodialysis sessions. Twenty-six healthy control subjects (10 men, 16 women; age, 52.5 ± 1.8

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years) also were tested. All individuals gave written informed consent for participation in the study. Plasma aliquots were quickly separated and frozen at −30°C for analysis by batches.

Analytical methods

Plasma total Hcy and Cys concentrations were determined by reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) thiol derivatives of Hcy and Cys using a modification of the method of Toyo′oka and Imai [11] with dithioerythritol as the reducing agent.

Free thiols were determined in both a filtered fraction and an acid-soluble fraction. Plasma samples were passed through a filter with a 10,000 D cutoff (ULTRACENT-10; Tosoh, Tokyo, Japan) to obtain the filtered fraction. The acid-soluble fraction was obtained after protein precipitation with sulfosalicylic acid (SSA). One milliliter of the plasma was mixed immediately with 0.1 mL 30% SSA, kept at 4°C for 1 hour, and centrifuged at 2500 *g* at 4°C for 20 minutes. The supernatant was collected and neutralized with NaOH. Free forms of total Hcy and total Cys were measured in the filtered fraction and in the SSA-treated fraction (acid-soluble Hcy, acid-soluble Cys). We confirmed that the pretreatment with the filter or acid precipitation did not affect the determination of these thiols using standard solution in saline.

Statistical analysis

Results are reported as mean ± SEM. Normality of distribution was confirmed by the Kolmogorov-Smirnov test. Results were analyzed using Student *t* test for paired and unpaired data. A P value <0.05 was considered significant. Statistics were performed on a DOS/V computer with StatView for Windows (Version 5.0, SAS institute, Cary, NC, USA).

RESULTS

Concentrations of total Hcy, filtered Hcy, and acid-soluble Hcy were significantly higher in prehemodialysis than control samples, and decreased 30.1 ± 2.6%, 53.7 ± 4.3%, and 38.5 ± 4.0%, respectively, after hemodialysis. However, total Hcy and acid-soluble Hcy posthemodialysis remained higher than for controls. Posthemodialysis filtered Hcy was significantly lower than acid-soluble Hcy ($P = 0.00036$) (Fig. 1A).

Most strikingly, filtered Cys was very small in controls, but was remarkably elevated in prehemodialysis samples. The free-to-total (F/T) ratio for Cys was only 1.6 ± 0.3% in controls, but was 40.9 ± 2.7% in prehemodialysis samples. The filtered Cys concentration was significantly lower than acid-soluble Cys in controls, but was higher than acid-soluble Cys in prehemodialysis samples (Fig. 1B). Total Cys, filtered Cys, and acid-soluble Cys were significantly higher in prehemodialysis than control samples, and decreased 63.6 ± 1.1%, 76.8 ± 1.9%, and 49.8 ± 3.7%, respectively, after hemodialysis. While total Cys and acid-soluble Cys declined to the control range, filtered Cys in posthemodialysis samples was significantly greater than in control samples.

The reduction rate for filtered Hcy or filtered Cys was significantly larger than that for acid-soluble Hcy ($P = 0.049$) or acid-soluble Cys ($P < 0.000015$).

DISCUSSION

The present study demonstrated that the unbound form of Cys in a filtered fraction from control plasma was much smaller than estimated in previous reports using an acid-soluble fraction. The difference in F/T ratio calculated for Cys in the filtered fraction between control and prehemodialysis samples also was remarkable (1.6 ± 0.3% vs. 40.9 ± 2.7%), although the F/T ratio for Hcy was comparable between control and hemodialysis groups. This is the first report where thiols were measured in filtered plasma fractions in control as well as hemodialysis subjects.

All previous reports concerning the free forms of Hcy and Cys used the acid-soluble fraction for the determination. However the F/T ratio of Hcy and Cys varied between reports. We speculated that this variation could have arisen from differences in acidifying agents and in methods of thiol determination. However, in both this and previous reports, the F/T ratio for acid-soluble Cys was greater than that for acid-soluble Hcy in control samples (36.6 ± 1.0 vs. 8.4 ± 1.4%) as well as prehemodialysis samples (25.2 ± 1.4 vs. 15.8 ± 1.0%).

The striking difference between filtered Cys and acid-soluble Cys in control samples shows that acid precipitation may change the binding constant between plasma proteins and thiols, liberating Cys from protein. In estimating the acid-soluble fraction, one should consider that the acid precipitation can fail to precipitate low-molecular-weight proteins. In considering glomerular filtration or solute movement across a dialysis membrane, we presumed that acid precipitation may not be an appropriate method for preparing samples to determine free thiol quantities in the estimation of solute movement across the dialysis membrane.

We could not rule out of the possibility that hemodialysis patients accumulated low-molecular-weight proteins in the plasma, which were bound with substantial amount of thiols and leaked from the filter. However, albumin is the most abundant protein in plasma and albumin-Cys[34] accounts for the bulk of thiols. Therefore, it is reasonable to think that filterable fractions of thiols could be unbound form.

The ease of oxidation of Cys and Hcy results in a

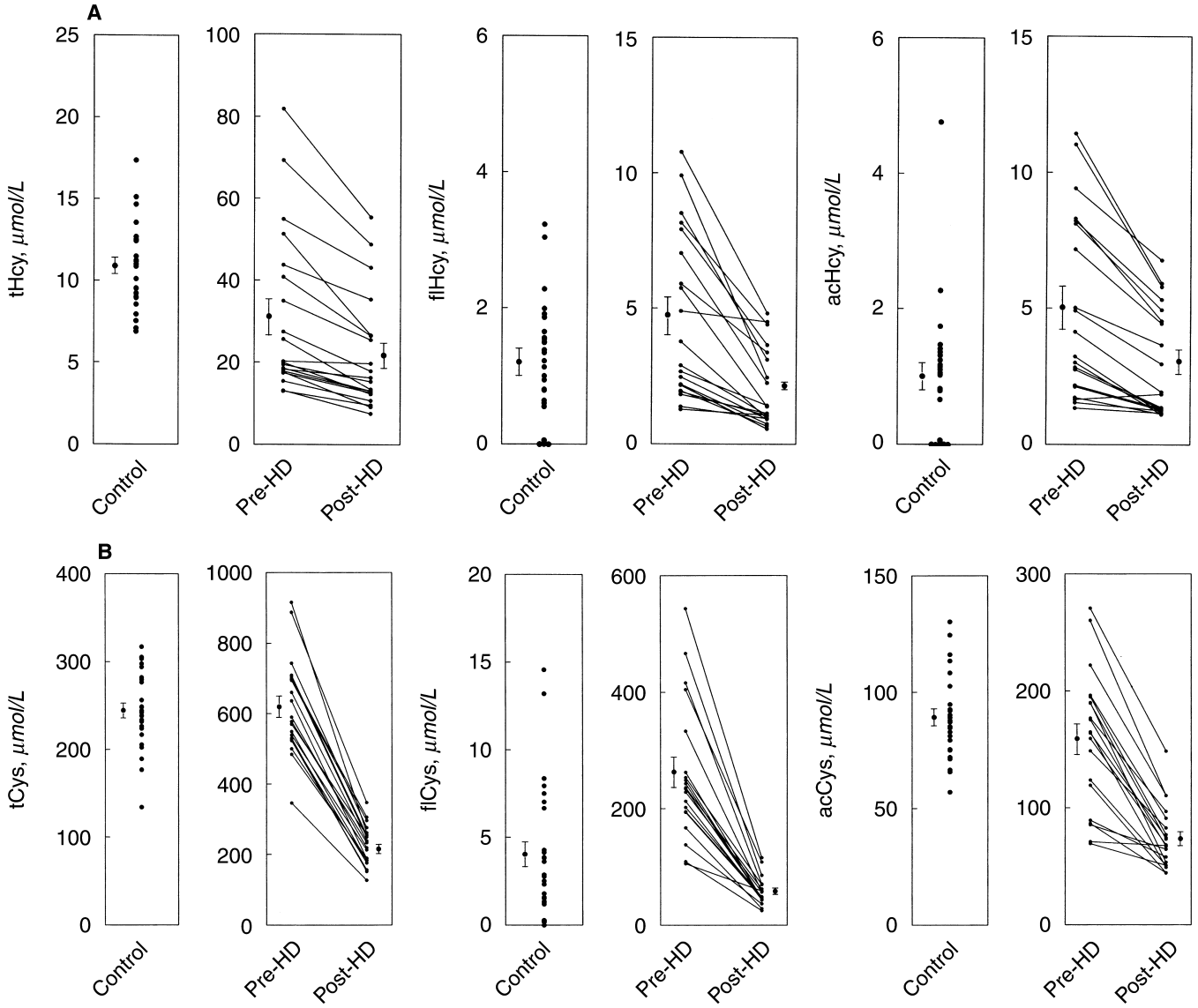

Fig. 1. Homocysteine (Hcy) and cysteine (Cys) in the total (t), filtered (fl), and acid soluble (ac) fraction of plasma before hemodialysis (pre-HD) and after hemodialysis (post-HD. (*A*) shows tHcy, flHcy and acHcy, and (*B*) shows tCys, flCys and acCys. In controls, flHcy and acHcy were similar, while flCys was much smaller than in acCys. In pre-HD samples, flCys was more than 60 times as abundant as in control samples. The T bars represent standard error.

variety of disulfide forms in vivo [12, 13], including low-molecular-weight disulfides and mixed disulfides associated with plasma protein, mainly albumin. The filterable fractions of thiols contain reduced-form and low-molecular-weight disulfides. In the present study, the F/T ratio of filtered Cys (free Cys) in prehemodialysis samples was significantly greater than in controls, while the F/T ratios of filtered Hcy in control and prehemodialysis samples were similar (12.3 ± 1.7% vs. 15.2 ± 1.2%). The significant difference of the F/T ratio between filtered Cys and filtered Hcy (free Hcy) was also observed. We presumed that the binding of Hcy to albumin was not affected in the uremic plasma, but a variety of qualitatively or quantitatively abnormal substances accumulating in uremia might compete with Cys for binding sites on albumin. The presumption might explain the difference of F/T ratio of filtered Cys before and after hemodialysis, as well as the difference of the reduction rate of filtered Hcy and filtered Cys in the hemodialysis session.

Thiols in the filtered fraction, whether reduced or disulfide, might be taken up into cells and disturb cellular functions, while protein-bound forms of thiols might be relatively stable. The present study demonstrated that the filterable form of Cys was over 60 times higher in hemodialysis (259.2 ± 26.2 μmol/L) than in controls (4.1 ± 0.7 μmol/L), which might be harmful to patients with end-stage renal disease and relevant to the high incidence of cardiovascular disease in hemodialysis.

CONCLUSION

In the present study, we analyzed unbound thiol in filtered and acid-soluble fractions of plasma, and compared them in control and hemodialysis patients. While filtered Hcy was comparable to acid-soluble Hcy, filtered Cys was very different from acid-soluble Cys. Strikingly, in controls, filtered Cys was found to be much smaller than acid-soluble Cys, while rising remarkably in pre-hemodialysis samples. The unbound form of Cys in pre-hemodialysis samples was more than 60 times as abundant as in controls. We concluded that thiols in the filtered fraction can be used to estimate membrane transport across the dialysis membrane. Further investigation should be necessary for the clarification of the involvement of Cys in the oxidative stress in hemodialysis.

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REFERENCES

1. Robinson K, Gupta A, Dennis V, *et al*: Hyperhomocysteinemia confers an independent increased risk of atherosclerosis in end-stage renal disease and is closely linked to plasma folate and pyridoxine concentrations. *Circulation* 94:2743–2748, 1996
2. Moustapha A, Naso A, Nahlawi M, *et al*: Prospective study of hyperhomocysteinemia as an adverse cardiovascular risk factor in end-stage renal disease. *Circulation* 97:138–141, 1998
3. Mallamaci F, Zoccali C, Tripepi G, *et al*: Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. *Kidney Int* 61:609–614, 2002
4. Welch GN, Loscalzo J: Homocysteine and atherothrombosis. *N Engl J Med* 338:1042–1050, 1998
5. Mudd SH, Finkelstein JD, Refsum H, *et al*: Homocysteine and its disulfide derivatives: a suggested consensus terminology. *Arterioscler Thromb Vasc Biol* 20:1704–1706, 2000
6. Hoffer LJ, Robitaille L, Elian KM, *et al*: Plasma reduced homocysteine concentrations are increased in end-stage renal disease. *Kidney Int* 59:372–377, 2001
7. Mansoor MA, Svardal AM, Ueland PM: Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal Biochem* 200:218–229, 1992
8. Tyagi SC: Homocyst(e)ine and heart disease: pathophysiology of extracellular matrix. *Clin Exp Hypertens* 21:181–198, 1999
9. Hultberg B, Andersson A, Arnadottir M: Reduced, free and total fractions of homocysteine and other thiol compounds in plasma from patients with renal failure. *Nephron* 70:62–67, 1995
10. Suliman ME, Anderstam B, Lindholm B, Bergstrom J: Total, free, and protein-bound sulphur amino acids in uraemic patients. *Nephrol Dial Transplant* 12:2332–2338, 1997
11. Toyo'oka T, Imai K: High performance liquid chromatography and fluorometric detection of biologically important thiols, derivatized with ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate (SBD-F). *J Chromatogr* 282:495–500, 1983
12. Ueland PM, Mansoor MA, Guttormsen AB, *et al*: Reduced, oxidized and protein-bound forms of homocysteine and other aminothiols in plasma comprise the redox thiol status–a possible element of the extracellular antioxidant defense system. *J Nutr* 126:1281–1284, 1996
13. Mansoor MA, Ueland PM, Aarsland A, *et al*: Redox status and protein binding of plasma homocysteine and other aminothiols in patients with homocystinuria. *Metabolism* 42:1481–1485, 1993